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Multiple Sites of Proteolytic Cleavage to Release Soluble Forms of Hepatocyte Growth Factor Activator Inhibitor Type 1 from a Transmembrane Form[1]

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Hepatocyte growth factor activator inhibitor type 1 (HAI-1) is a Kunitz-type serine protease inhibitor, which was identified as a potent inhibitor of hepatocyte growth factor (HGF) activator from the conditioned medium of a human carcinoma cell line. HGF activator is a blood coagulation factor XII-like serine protease that is responsible for proteolytic activation of the inactive single chain precursor of HGF in injured tissues. The predicted sequence of the primary translation product of HAI-1, which has a hydrophobic sequence in its COOH-terminal region, suggested that HAI-1 is first produced in a membrane-associated form. In this study, we identified a transmembrane form of HAI-1 integrated in the plasma membrane of cultured cells using a monoclonal antibody against HAI-1. We also identified several soluble forms of HAI-1 in the conditioned medium of the cells, indicating that multiple sites are present in the transmembrane form of HAI-1 at which proteolytic cleavage releases the extracellular domain. At least two proteases, one of which is a metalloprotease, appear to be responsible for the release. Further, the soluble forms of HAI-1 have different inhibitory activity against HGF activator. These findings suggest that proteolytic processing plays important roles in regulation of the inhibitory activity of HAI-1.

Key words: ectodomain shedding, hepatocyte growth factor, hepatocyte growth factor activator inhibitor, Kunitz-type inhibitor, metalloprotease.

Hepatocyte growth factor activator inhibitor type 1 (HAI-1) was initially identified as a potent inhibitor of hepatocyte growth factor (HGF) activator from the conditioned medium of a human stomach carcinoma cell line (*1*). A second type of HGF activator inhibitor (HAI-2) was subsequently identified from the same conditioned medium (*2*). Both HAI-1 and HAI-2 have been shown to be Kunitz-type serine protease inhibitors (*1, 2*). HAI-2 is identical to two independently isolated Kunitz-type inhibitors, placental bikunin, and the *kop* product (*3, 4*). HGF activator is a blood coagulation factor XII-like serine protease that is responsible for proteolytic activation of the inactive single chain precursor of HGF in injured tissues (*5-7*). The activated HGF appears to be involved in repair of the injured tissues (*8-11*). After tissue repair, proteolytic activation of HGF may be terminated by suppressing the HGF-converting activity of HGF activator to prevent further action of HGF. Because HAI-1 and HAI-2 efficiently inhibit the proteolytic activity of HGF activator *in vitro* (*1, 2*), they may suppress the HGF-converting activity of HGF activator in repaired tissues.

The primary translation product of HAI-1, which was predicted from the cDNA sequence, consists of 513 amino acid residues with a calculated molecular mass of 57 kDa including a NH_2-terminal signal peptide sequence of 35 amino acid residues (*1*). It contains two Kunitz domains and an LDL receptor-like domain between them. In addition, it has a hydrophobic region of 23 amino acids at the COOH-terminal region, suggesting that HAI-1 is first produced in a membrane-associated form (*1*). Analysis of HAI-1 purified from the conditioned medium of a human carcinoma cell line MKN45 by SDS-PAGE under reducing conditions revealed two molecules with molecular masses of 39 and 40 kDa (*1*). The heterogeneity of the purified protein on the gel may be caused by a difference in the COOH-terminal sequence or in glycosylation. The molecular mass of HAI-1 was decreased by *N*-glycosidase treatment, indicating that HAI-1 is an *N*-glycosylated protein (*2*). Comparison of the NH_2-terminal amino acid sequence and molecular mass of the protein purified from the conditioned medium with

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Abbreviations: CHAPS, 3-((3-cholamidopropyl)dimethylammonio)-1-propanesulfonic acid; FBS, fetal bovine serum; FITC, fluorescein-5-isothiocyanate; HAI-1, hepatocyte growth factor activator inhibitor type 1; HGF, hepatocyte growth factor; HRP, horseradish peroxidase; mAb, monoclonal antibody; PBS, phosphate-buffered saline; TCA, trichloroacetic acid.

those of the predicted primary translation product revealed that the soluble form of HAI-1 is derived from the NH_2-terminal region of the primary translation product by proteolytic cleavage, and contains only a Kunitz domain (*1*).

HGF activator is produced and secreted by parenchymal liver cells (*12*) and circulates in the blood as an inactive zymogen (*6*). In injured tissues, the zymogen is converted to an active serine protease that appears to be associated with the extracellular matrix (*7*). These observations suggest that the soluble form of HAI-1 is responsible for inhibiting the proteolytic activity of HGF activator. Thus, it is important to investigate proteolytic processing pathways to generate the soluble form of HAI-1. In this study, we prepared a monoclonal antibody against HAI-1 and analyzed molecular forms of HAI-1 in the producing cells and their conditioned medium using the antibody. We identified a transmembrane form of HAI-1 in the producing cells. We also identified several molecular forms of HAI-1 in the conditioned medium in addition to the previously identified molecules. Characterization of these molecules revealed the proteolytic processing pathways of HAI-1 in cultured cells.

MATERIALS AND METHODS

Materials—Cell lines and reagents were obtained as follows: human stomach carcinoma cell line MKN45 from IBL; human lung carcinoma cell line HLC-1 from the Department of Physiology, Keio University; trypsin, 1,10-phenanthroline, L-*trans*-epoxysuccinyl-leucylamide-(4-guanidino)-butane (E-64), pepstatin, iodoacetoamide, phenylmethylsulfonyl fluoride (PMSF), and 3,4-dichloroisocumarin (3,4-DCI) from Sigma; chymotrypsin from Miles Lab.; *N*-hydroxysulfosuccinimidobiotin (sulfo-NHS-biotin) from PIERCE; horseradish peroxidase (HRP) from Boehringer Mannheim GmbH; streptoavidin-agarose from Novagen; non-specific mouse IgG and fluorescein-5-isothiocyanate conjugated sheep IgG anti-mouse IgG (Anti mouse IgG-FITC) from Organo Teknika; 10K OMEGA membrane from FILTRON; C4 pack column from YMC; immunostaining HRP-1000 kit from Konica; DC protein assay kit from Bio-Rad; ECL Western blotting detection reagents from Amersham-Pharmacia Biotech. A monoclonal antibody (mAb) against HAI-1 was prepared as follows. A Balb/c mouse was immunized with HAI-1 purified from the conditioned medium of MKN45 cells (*1*). Spleen cells were removed from the mouse and fused with P3U1 myeloma. Hybridomas were screened for secretion of antibodies, and a hybridoma (C76-18 cells) was obtained.

Immunoblotting Analysis of HAI-1 Proteins in Cell Extract and Conditioned Medium—MKN45 cells were cultured in a 9-cm culture dish in eRDF medium containing 5% fetal bovine serum (FBS). At confluence, the medium was replaced by serum-free medium. After 2 days, the conditioned medium was harvested and used for immunoblotting analysis. To prepare cell extract, the cells were washed with cold 0.9% NaCl and treated with 1 ml of 10% trichloroacetic acid (TCA) for 15 min on ice. The TCA-insoluble fraction was pelleted by centrifugation. The pellet was thoroughly dissolved in 150 μl of 7 M urea containing 2% Triton X-100 and 5% 2-mercaptoethanol, then centrifuged. The supernatant was recovered and used for immunoblotting analysis. For acid or salt treatment, the cells were washed with cold 0.9% NaCl, then incubated on ice for 1 min with 10 ml of 50 mM acetic acid in 0.9% NaCl (pH 3.0) or with 10 ml of 1 M NaCl. After incubation, the cells were washed with 0.9% NaCl, and cell extract was prepared by TCA treatment as described above. For protease treatment, the cells were washed with cold 0.9% NaCl and incubated with 1 ml of 0.01% trypsin or 0.01% chymotrypsin in phosphate-buffered saline (PBS) at room temperature. After the protease treatment, the cell extract was prepared by TCA treatment as described above. Aliquots (40 μl) of the samples were analyzed by SDS-PAGE in the presence of 2-mercaptoethanol on a 12.5% gel. After SDS-PAGE, proteins were transferred to a polyvinylidene difluoride membrane. The membrane was incubated with HRP-conjugated C76-18 mAb and developed with an Immunostaining HRP-1000 kit or an enhanced chemiluminescence (ECL) Western blotting detection system.

Biotinylation of Cell Surface Proteins—MKN45 cells were washed with 10 mM sodium-borate (pH 8.8) containing 150 mM NaCl, then incubated with 3 ml of 100 μg/ml sulfo-NHS-biotin in the washing buffer for 10 min at room temperature. The reaction was quenched by the addition of NH_4Cl to 10 mM. The cells were washed with 50 mM Tris-HCl (pH 7.4) containing 25 mM KCl, 5 mM $MgCl_2$, and 1 mM EDTA, and treated with 1 ml of 10% TCA for 15 min on ice. The TCA-insoluble fraction was pelleted by centrifugation. The pellet was dissolved in 200 μl of 7 M urea containing 0.5% Triton X-100, then centrifuged. The supernatant (150 μl) was neutralized with 20 μl of 1.5 M Tris-HCl (pH 8.5) and incubated with 150 μl streptavidin-agarose resin at 4°C overnight. After centrifugation, an aliquot (60 μl) of the supernatant was analyzed by immunoblotting.

Immunocytostaining—MKN45 and HLC-1 cells were cultured in chamber 8-well glass slides in eRDF medium containing 5% FBS. At semi-confluence, the cells were washed with cold PBS containing 0.1% gelatin and 0.1% NaN_3 (washing buffer), and incubated with 200 μl of 50 μg/ml anti-HAI-1 mAb (C76-18) or 500 μg/ml control mouse IgG for 60 min on ice. After the incubation, the cells were washed with washing buffer and incubated with 200 μl of sheep IgG anti-mouse IgG-FITC for 30 min on ice. After the incubation, the cells were washed with washing buffer and visualized by use of a fluorescence microscope.

Affinity Purification of HAI-1—MKN45 cells were inoculated in roller bottle 850 and cultured in eRDF medium containing 5% FBS. At confluence, the medium was replaced by serum-free medium, and the cells further cultured for 2 days. After the culture, the conditioned medium (15 liters) was collected, concentrated to 300 ml with 10 K OMEGA membrane, and adjusted to 20 mM phosphate concentration with 500 mM sodium phosphate (pH 7.3). The concentrates were applied to a hydroxyapatite column (2.5×5 cm) pre-equilibrated with 20 mM sodium phosphate (pH 7.3) containing 150 mM NaCl. The flow-through fraction (about 350 ml) was adjusted to 300 mM NaCl and applied to a C76-18 mAb-immobilized resin (1×2 cm) pre-equilibrated with 20 mM sodium phosphate (pH 7.3) containing 300 mM NaCl. The resin was washed with the equilibration buffer, and HAI-1 proteins were eluted with 10 mM HCl. The eluate was neutralized immediately with

1 M Tris-HCl (pH 8.0). The purified proteins were resolved by SDS-PAGE (12.5% gel) under reducing and nonreducing conditions and stained with silver. For NH_2-terminal amino acid sequence determination, the eluate from the affinity column was subjected to reverse-phase high performance liquid chromatography (HPLC) on a C4 packed column. Protein concentration was determined by DC protein assay with bovine serum albumin as a standard.

Construction and Expression of a Plasmid Encoding a COOH-Terminally Truncated Form of HAI-1—A cDNA encoding a deletion mutant of HAI-1, which was truncated at the end of the second Kunitz domain, was constructed by PCR using the full-length cDNA as a template and two oligonucleotides, 5′-GGCCGCGGCCAACGTCACA-3′ and 5′-GCGCGGCCGCGACAAGACT-3′, as primers. The PCR product was digested with *Bgl*II and *Not*I, generating a 0.5 kbp fragment. The *Eco*RI and *Bgl*II fragment encoding the NH_2-terminal region of HAI-1 and the 0.5 kbp *Bgl*II-*Not*I fragment were ligated to the *Eco*RI and *Not*I site of a expression plasmid pME18S (*13*). The plasmid and pSV2 neo were cotransfected into CHO cells by the lipofectoamine method described by Derijard *et al.* (*14*). After transfection, the cells were cultured in eRDF medium containing 0.4 mg/ml G418 and 10% FBS. G418-resistant colonies were selected and screened for expression of the truncated HAI-1 by immunoblotting using mAb of HAI-1.

Assay for Inhibitory Activity against HGF Activator—Various concentrations of HAI-1 were incubated with 9 ng of HGF activator in 45 μl of 50 mM Tris-HCl (pH 8.0) containing 0.1% CHAPS and 150 mM NaCl at 37°C for 20 min. The mixtures were added to 5 μl of 1 mg/ml single chain HGF and further incubated for 2 h. The mixtures were then analyzed by SDS-PAGE under reducing conditions. The gel was stained with Coomassie Brilliant Blue and scanned by use of a Flying-Spot Scanner CS-9000 (Shimadzu). The inhibitory activity of HAI-1 was estimated by calculating the ratio of the remaining single-chain form to total HGF.

Assay for Binding of HAI-1 to HGF Activator—HCL-1 cells were cultured in 6-well plates in eRDF medium containing 5% FBS. At confluence, the medium was replaced by serum-free medium, and the cells were cultured for one day. The cells were washed with PBS, then incubated in medium containing each protease inhibitor at 37°C for 1.5 h. After incubation, the conditioned medium of cells treated with protease inhibitors other than serine protease inhibitors was directly assayed for binding to HGF activator. The conditioned medium of cells treated with serine protease inhibitors was assayed after the inhibitors were removed by ultrafiltration. Fifty microliters of a monoclonal antibody against HGF activator (A23) (10 μg/ml) was adsorbed to a 96-well assay plate, and blocked with PBS containing 0.1% gelatin (blocking buffer). Fifty microliters of the 34-kDa active form of HGF activator (1 μg/ml) in blocking buffer was added to the plate and incubated at 4°C for 24 h. The assay samples containing HAI-1 were diluted with 50 μl of 50 mM Tris-HCl (pH 8.0) containing 0.1% gelatin and 150 mM NaCl (reaction buffer), and incubated with HGF activator on the plate at 4°C for 24 h. Fifty microliters of the HRP-conjugated anti-HAI-1 mAb (10 μg/ml) in reaction buffer was used to detect the complex of HGF activator and HAI-1. The HAI-1 protein previously purified from the conditioned medium of MKN45 cells (*1*) was used as a standard of the binding activity. Results are expressed as the mean ± SD (n=4).

RESULTS

Identification of a Transmembrane Form of HAI-1—To detect the primary translation product of HAI-1, which appears to be a membrane-associated form, we first analyzed HAI-1 molecules in the extract of MKN45 cells by immunoblotting using a monoclonal antibody against HAI-1 (C76-18) and compared them with those in the conditioned medium. A 66-kDa band was detected in the cell extract (Fig. 1A, lane 2), whereas four bands (58, 48, 40, and 39 kDa) were detected in the conditioned medium (Fig. 1A, lane 1). The treatment of cells with a low pH buffer or a high salt solution did not change the amount of the 66-kDa band in the cell extract (Fig. 1A, lanes 3 and 4). On the other hand, the treatment of cells with trypsin or chymotrypsin decreased the amount of the 66-kDa band, which almost disappeared after treatment for 5 min with either protease (Fig. 1, B and C). These results, together with the fact that the primary translation product of HAI-1 has a hydrophobic sequence in the COOH-terminal region, indicate the presence of a transmembrane form of HAI-1 with a molecular mass of 66 kDa in the plasma membrane.

Because the NH_2-terminal region of the primary translation product of HAI-1 is released in the conditioned medium (*1*), this region is most likely to correspond to the large extracellular domain of the transmembrane form. To confirm the presence of the extracellular region of the 66-kDa HAI-1, we examined whether HAI-1 on the cell surface was biotinylated. Cells were treated with a biotinylation reagent, and cell extract was prepared. After adsorption of the biotinylated proteins in the extract with streptavidin-resin, the nonadsorbed proteins were analyzed by immunoblotting (Fig. 2). The 66-kDa band in cell extract became undetectable by biotinylation followed by streptavidin-resin adsorption (Fig. 2, lane 4). Streptavidin-

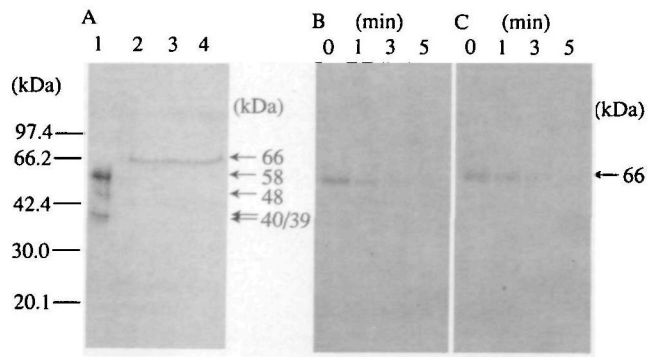

Fig. 1. **Immunoblotting analysis of HAI-1 in cell extract and conditioned medium.** Proteins in the cell extract and conditioned medium of MKN45 cells were separated by SDS-PAGE (12.5% polyacrylamide) under reducing conditions and transferred to a polyvinylidene difluoride membrane. The membrane was incubated with HRP-conjugated mAb, and developed with a Immunostaining HRP-1000 kit. A, lane 1, HAI-1 in the conditioned medium; lane 2, HAI-1 in the cell extract; lane 3, HAI-1 in the extract of cells treated with a low pH buffer; lane 4, HAI-1 in the extract of cells treated with a high salt solution. Molecular mass markers are shown in kilodaltons on the left. B and C, HAI-1 in the extract of cells treated with trypsin and chymotrypsin for the indicated time, respectively.

resin adsorption or biotinylation alone did not change the amount of the 66-kDa band (Fig. 2, lanes 2 and 3). These results show that the 66-kDa HAI-1 was biotinylated, confirming the presence of the extracellular region of the 66-kDa HAI-1.

To detect the transmembrane form of HAI-1 in living cells, we reacted MKN45 cells and HLC-1 cells with the anti-HAI-1 mAb, and cell-bound mAb was detected with sheep IgG anti-mouse IgG-FITC. Immunostained HAI-1 on cell surfaces was detected by the anti-HAI-1 mAb, but not by a nonspecific mouse IgG (Fig. 3). These results indicate that HAI-1 proteins are present on the plasma membrane of living cells.

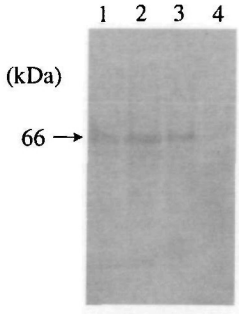

Fig. 2. **Immunoblotting analysis of biotinylated HAI-1 on cell surface.** Lane 1, extract of untreated MKN45 cells; lane 2, cell extract treated with a streptavidin-resin; lane 3, extract of the cells treated with a biotinylation reagent; lane 4, extract prepared from cells treated with a biotinylation reagent, followed by streptavidin-resin adsorption. Immunoblotting analysis was performed as described in Fig. 1.

Multiple Forms of HAI-1 in the Conditioned Medium—The molecular mass of HAI-1 purified from the conditioned medium of MKN45 cells based on the inhibitory activity against HGF activator was 40 and 39 kDa on the gel of SDS-PAGE under reducing conditions (*1*). The heterogeneity may be caused by a difference in the COOH-terminal sequence or in glycosylation. By the immunoblotting analysis, four bands of 58, 48, 40, and 39 kDa were detected in the conditioned medium of MKN45 cells (Fig. 1A, lane 1). These bands were not detected in the cell extract (Fig. 1A, lane 2). Further incubation of the conditioned medium in the absence of the cells or culture of the cells for different periods did not change the ratio of these bands (data not shown). These results suggest that the proteolytic processings to produce these soluble forms occur independently on the plasma membrane. The four forms of HAI-1 were purified by column chromatography on an anti-HAI-1 mAb immobilized resin. SDS-PAGE of the purified HAI-1 proteins under reducing conditions revealed four bands which correspond to those detected by the immunoblotting analysis (Fig. 4B, lane 2). SDS-PAGE under nonreducing conditions showed two bands of 48 and 38 kDa (Fig. 4A, lane 2). The previously purified HAI-1 corresponds to the bands of 40 and 39 kDa under reducing conditions and 38 kDa under nonreducing conditions, because they migrated

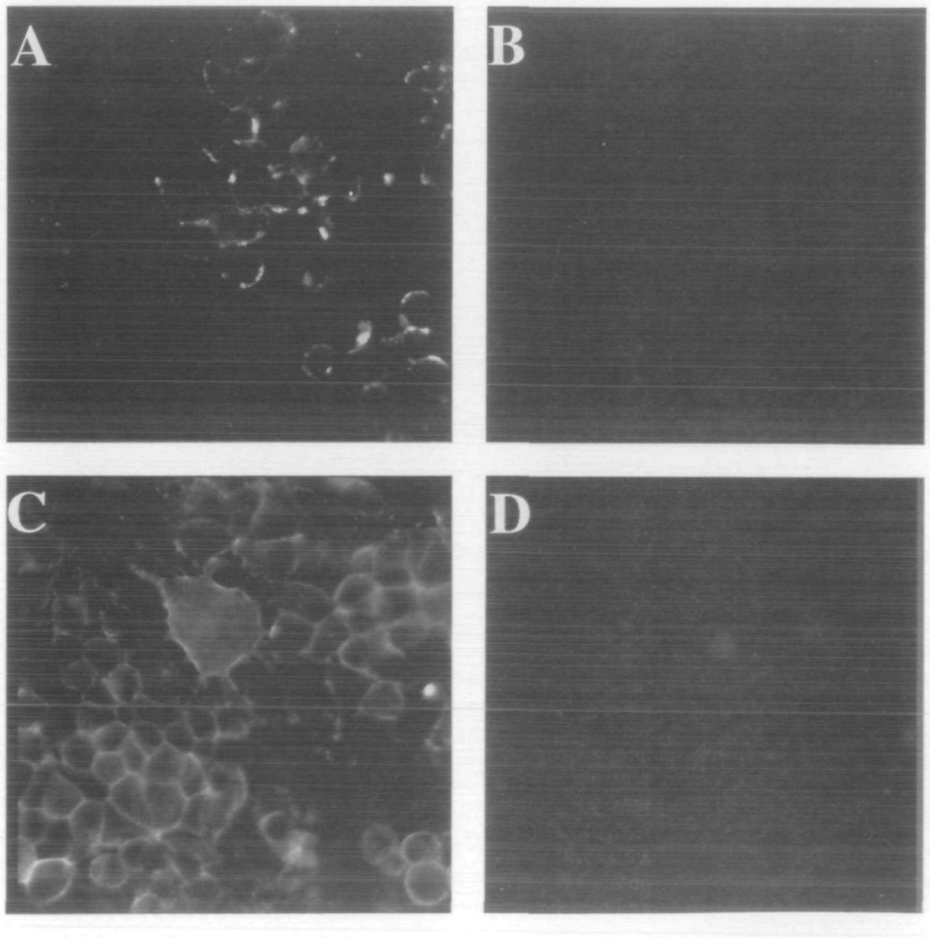

Fig. 3. **Immunocytostaining of cultured cells with anti-HAI-1 mAb.** MKN45 (A and B) and HLC-1 (C and D) cells were incubated with anti-HAI-1 mAb (A and C) or control mouse IgG (B and D). The cells were incubated with sheep IgG anti-mouse IgG-FITC and visualized by use of a fluorescence microscope.

to the same positions on the gels of SDS-PAGE (Fig. 4, A and B). The 48-kDa band under nonreducing conditions was separated into two bands of 58 and 48 kDa under reducing conditions, suggesting that the 48-kDa band under reducing conditions is derived from the 58-kDa form by endoproteolytic cleavage and linked to a small fragment by a disulfide bond.

The 58-kDa HAI-1 protein was isolated by HPLC of the immunoaffinity-purified HAI-1 proteins. Its NH_2-terminal amino acid sequence was determined to be identical to that of the previously purified 40/39-kDa HAI-1. We tried to determine the COOH-terminal amino acid sequence by the method of Nokihara *et al.* (*15*), but it was not successful. To circumvent the problem, we constructed cDNA encoding a deletion mutant of HAI-1 which is truncated at the COOH-terminal end of the second Kunitz domain and stably transfected it into CHO cells. The truncated form of HAI-1 was purified from the conditioned medium by column chromatography on an anti-HAI-1 mAb immobilized resin and compared with the 58-kDa protein by SDS-PAGE (Fig. 5). The 58-kDa protein migrated to almost the same position as the truncated form of HAI-1. These results suggest that the 58-kDa HAI-1 includes two complete Kunitz domains.

The 40/39-kDa HAI-1, which contains only one Kunitz domain, forms an equimolar complex with HGF activator (*1*). The inhibitory activity of the 58-kDa HAI-1 against HGF activator was compared with that of the 40/39-kDa HAI-1 (Fig. 6). The concentrations of the 58-kDa and 40/39-kDa HAI-1 for 50% inhibition were 1,000 and 200 ng/ml, respectively. Adjusted for their molecular masses, the inhibitory activity of the 58-kDa HAI-1 was about 30% of that of the 40/39-kDa HAI-1.

Effects of Protease Inhibitors on the Release of Soluble Forms of HAI-1 from Cultured Cells—The HAI-1 proteins in the conditioned medium of cultured cells are produced from the transmembrane form by proteolytic processing.

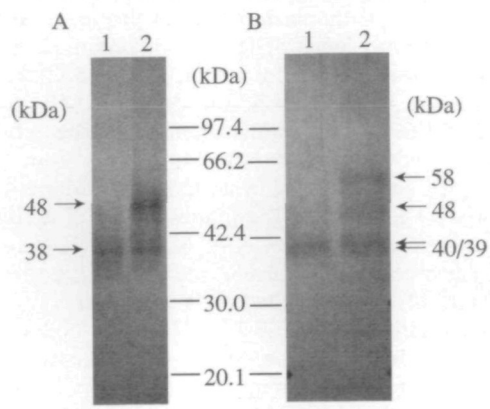

Fig. 4. **SDS-PAGE analysis of HAI-1 purified from the conditioned medium of MKN45 cells.** Purified HAI-1 proteins were separated by SDS-PAGE (12.5% polyacrylamide) under nonreducing (A) and reducing (B) conditions and stained with silver. Lane 1, HAI-1 proteins purified as described previously (*1*); lane 2, HAI-1 proteins purified by immunoaffinity chromatography.

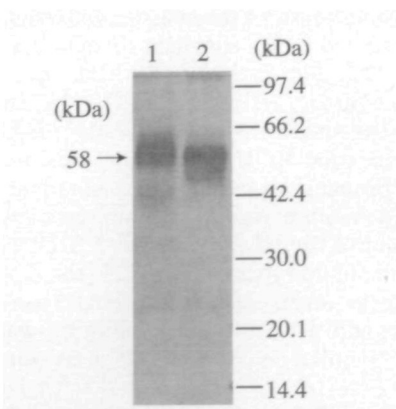

Fig. 5. **Comparison of the 58-kDa HAI-1 with the COOH-terminally truncated HAI-1 by SDS-PAGE.** The purified 58-kDa HAI-1 (lane 1) and truncated HAI-1 (lane 2) were separated by SDS-PAGE (12.5% polyacrylamide) under reducing conditions and stained with silver.

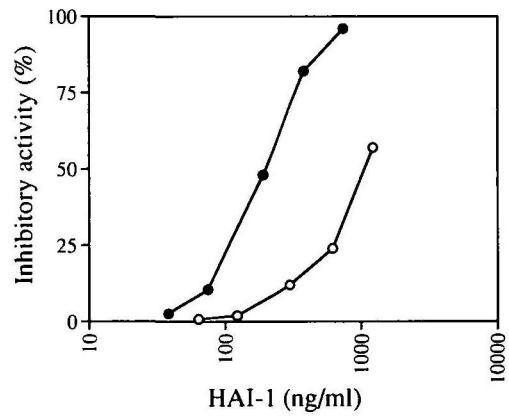

Fig. 6. **Dose-dependence of the inhibitory activity of the 58- and 40/39-kDa HAI-1 against HGF activator.** Various concentrations of the 58-kDa (open circle) and 40/39-kDa (closed circle) forms were incubated with HGF activator. Then single-chain HGF was added, and the mixture further incubated. The reaction products were separated by SDS-PAGE. The inhibitory activity of HAI-1 was determined as the ratio of the remaining single-chain HGF to total HGF and plotted against HAI-1 concentration.

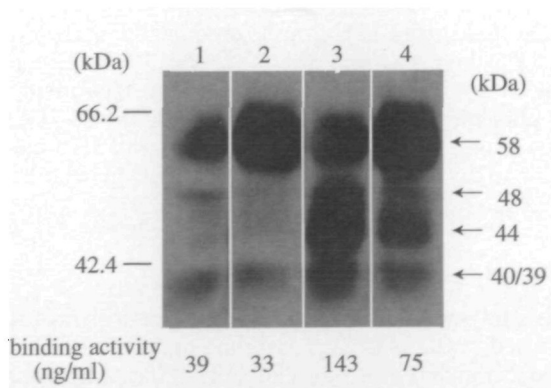

Fig. 7. **Immunoblotting analysis of HAI-1 proteins in the conditioned medium of cells treated with metal chelators.** HLC-1 cells were incubated for 1.5 h without (lane 1) or with 1,10-phenanthroline (lane 2), EDTA (lane 3), or both (lane 4). HAI-1 proteins in the condition medium were separated by SDS-PAGE (12.5% polyacrylamide) under reducing conditions and transferred to a polyvinylidene difluoride membrane. The membrane was incubated with HRP-conjugated mAb and developed by ECL Western blotting detection system. The binding activity of HAI-1 to HGF activator in the conditioned medium is shown below the immunoblot.

To examine the proteases responsible for the processing, effects of protease inhibitors on the production of the soluble forms of HAI-1 were investigated. HLC-1 cells were used for this assay, because the cells produce a higher level of HAI-1 proteins than MKN45 cells. First, the binding activity of HAI-1 to HGF activator was measured in the conditioned medium of HLC-1 cells that were incubated with various protease inhibitors. Cysteine protease inhibitors (E-64 and iodoacetoamide), an aspartic protease inhibitor (pepstatin), and serine protease inhibitors (PMSF and 3,4-DCI) had no effect on the release of the binding activity of HAI-1. The binding activity was slightly decreased (33 ± 1.3 ng/ml) by treatment with 1,10-phenanthroline, a chelator of heavy metals, which may be an inhibitor of metalloproteases, when compared to the binding activity (39 ± 0.6 ng/ml) in the conditioned medium of untreated cells. In contrast, treatment with EDTA markedly increased the binding activity (143 ± 9.0 ng/ml) of HAI-1. A similar level of increase was observed on treatment with EGTA, a favorable chelator of Ca^{2+}. The binding activity of HAI-1 enhanced by EDTA was decreased (75 ± 2.7 ng/ml) by treatment with 1,10-phenanthroline.

Next, the molecular forms of HAI-1 in the conditioned medium of the cells treated with 1,10-phenanthroline, EDTA or both were analyzed by immunoblotting (Fig. 7). As in the conditioned medium of MKN45 cells, the four forms of HAI-1, 58, 48, 40, and 39 kDa, were detected in the conditioned medium of untreated HLC-1 cells (Fig. 7, lane 1), although the 40- and 39-kDa forms were not well separated on this gel. However, the level of the 48-kDa form was much lower than that in the conditioned medium of MKN45 cells. The levels of the 48- and 40/39-kDa forms were decreased by treatment with 1,10-phenanthroline. In contrast, the level of the 58-kDa form was markedly increased by the treatment (Fig. 7, lane 2). The treatment of EDTA increased the levels of all these forms. In addition, a 44-kDa form was newly produced, and its level was higher than those of other forms (Fig. 7, lane 3). The 44-kDa band under reducing conditions migrated to the position of 42 kDa under nonreducing conditions (data not shown), indicating that the 44-kDa form is produced in a single-chain form. The levels of the 48-, 44-, and 40/39-kDa forms enhanced by EDTA were decreased by treatment with 1,10- phenanthroline, whereas the level of the 58-kDa form was increased by treatment with EDTA and 1,10-phenanthroline to the level by treatment with 1,10-phenanthroline alone (Fig. 7, lane 4).

DISCUSSION

In this study, we identified a transmembrane form of HAI-1 integrated in the plasma membrane of cultured cells. The primary translation product of HAI-1 has a hydrophobic sequence in the COOH-terminal region (*1*). This sequence is most likely to be responsible for the transmembrane form of HAI-1. We also identified several soluble forms of HAI-1 in the conditioned medium of the cells. Analysis of their molecular structures indicated that all the soluble forms are generated from the NH_2-terminal region of the transmembrane form by proteolytic processing.

Two major soluble forms of HAI-1 (58 and 40/39 kDa) are released into the conditioned medium of untreated cultured cells. The 40/39-kDa form was previously purified from the conditioned medium (*1*). The 58-kDa form was not purified by the previous purification procedure, probably because it has relatively weak activity. In this study, the 58-kDa form was readily purified by the immunoaffinity chromatography. The 58-kDa form appears to be produced by cleavage at a site between the second Kunitz domain and the transmembrane domain, and thus contains two complete Kunitz domains. On the other hand, the 40/39-kDa form is produced by cleavage further upstream, probably between the first Kunitz domain and the LDL receptor-like domain, and contains one Kunitz domain. The 40/39-kDa form has a potent inhibitory activity against HGF activator, whereas the 58-kDa form shows much weaker activity, suggesting that a region in the COOH-terminal structure of the 58-kDa form interferes with the binding of HGF activator to the active site of the first Kunitz domain. Recently, the high-resolution crystal structure of bikunin, a serine protease inhibitor with two Kunitz domains, has been determined (*16*). The two Kunitz domains of bikunin pack closely together. The loop responsible for protease recognition of the first domain is unobstructed by the remaining protein. However, the loop of the second domain is close to the first domain, and thus protease binding is affected by the presence of the first domain. The second domain of bikunin inhibits factor Xa, plasma kallikrein, and tryptase, while the intact bikunin does not (*17, 18*). The presence of the first domain may abolish the binding of these proteases to the active site of the second domain (*16*). Similarly, the presence of the second Kunitz domain in the 58-kDa HAI-1 may affect the binding of HGF activator to the first domain. Thus, release of the COOH-terminal region by proteolytic processing could regulate the inhibitory activity of HAI-1 against HGF activator.

A small fraction of the 58-kDa HAI-1 is endoproteolytically cleaved in the untreated cells to generate a 48-kDa fragment and a small fragment which are linked by a disulfide bond. It is probable that the endoproteolytic cleavage precedes the cleavage to produce the 58-kDa form, because the ratio of the 48-kDa band to the 58-kDa band was not changed by incubation of the conditioned medium in the absence of the cells. Considering its molecular mass, the 48-kDa fragment appears to be produced by cleavage that occurs within the second Kunitz domain of HAI-1. The Kunitz domain contains three disulfide bonds. One of them may be involved in the disulfide linkage between the two fragments. The heterodimeric 58-kDa form might obtain the full inhibitory activity of HAI-1 by conformational change of the single-chain form of the 58-kDa molecule. Since purification of the molecule was not successful, however, probably because of its small amount in the conditioned medium, its activity remains to be determined.

Treatment of the HLC-1 cells with EDTA increased the levels of all soluble forms of HAI-1 (the single-chain and heterodimeric forms of 58-kDa HAI-1 and 40/39-kDa HAI-1). In addition, a 44-kDa molecule was newly produced. The binding activity to HGF activator in the conditioned medium of the cells treated with EDTA was increased about 3.5-fold over that in the conditioned medium of untreated cells. This increased activity may be attributed to not only the 58- and 40/39-kDa molecules but also the newly produced 44-kDa molecule. The increased binding activity was also observed in the conditioned medium of the

cells treated with EGTA, suggesting that chelating a calcium ion in the transmembrane form of HAI-1 increases the susceptibility to cleavage by proteases. A cysteine-rich domain of the low-density lipoprotein receptor (LDLR) has been shown to contain a calcium ion coordinated by acidic residues that lie at the COOH-terminal end of the domain (*19*). HAI-1 has a cysteine-rich domain similar to that in LDLR. Further, acidic residues are well conserved in the domain of HAI-1 (*1*), suggesting that the domain contains a calcium ion. Chelating the calcium ion may disrupt the highly organized structure of the domain and lead to its susceptibility to attack by proteases. Because a high level of the 44-kDa soluble form of HAI-1 was released in the conditioned medium of the cells treated with EDTA, the site of cleavage to release the 44-kDa HAI-1 is likely to be within the cystein-rich domain.

There are at least three sites of cleavage to release the extracellular domain of HAI-1. Cleavage at two of these sites was inhibited in the presence of a heavy metal chelator, 1,10-phenanthroline, suggesting that a metalloprotease(s) is responsible for these cleavages. EDTA could chelate heavy metal ions, but did not show the inhibitory effect. This may be due to the stronger affinity of EDTA to a calcium ion than to heavy metal ions. The extracellular domains of numerous transmembrane proteins are proteolytically released from the cell surface, a process known as ectodomain shedding. Cleavage at two sites (α and β sites) has been shown to lead to the release of two soluble forms of β-amyloid precursor protein (*20*). Several proteases are known to be involved in the process. Tumor necrosis factor-α (TNF-α) converting enzyme (TACE) and ADAM10 were identified as proteases responsible for the shedding of the extracellular domain of TNF-α (*21-23*). TACE has been shown to be also responsible for shedding of the extracellular domains of a TNF receptor, the L-selectin adhesion molecule, transforming growth factor-α, and β-amyloid precursor protein (*24-26*). Matrix Metalloprotease-3 releases a soluble form of heparin-binding epidermal growth factor-like growth factor from its transmembrane form (*27*). Because the activities of these proteases are blocked by metalloprotease inhibitors (*23, 28*), some of them may be responsible for releasing the extracellular domain of HAI-1. The use of specific metalloprotease inhibitors would probably help identify the metalloproteases involved. The cleavage to release the 58-kDa HAI-1 was not inhibited by 1,10-phenanthroline. The level of the 58-kDa HAI-1 was rather increased in the presence of the inhibitor. The increased level may partly result from the inhibition of the cleavage at the other sites. The cleavage to release the 58-kDa HAI-1 occurs between the second Kunitz domain and the transmembrane domain. A dibasic amino acid sequence (Arg^{437}-Arg^{438}), which is a possible cleavage site of the Kex family of serine proteases (*29*), is present in the region (*1*). Thus, a member of the family may be responsible for releasing the 58-kDa HAI-1.

HAI-1 is thought to act on HGF activator extracellularly, because the active form of HGF activator is associated with the extracellular matrix in injured tissue (*7*). Thus, only soluble forms of HAI-1 could be involved in inhibiting HGF activator. There are multiple cleavage sites to release the soluble forms of HAI-1 in cultured cells. At least two proteases appear to be involved in the cleavages, and calcium ions modulate the susceptibility of the HAI-1 protein to attack by the proteases probably by changing its conformation. Moreover, the soluble forms of HAI-1 have different inhibitory activity against HGF activator. Thus, not only identification of proteases responsible for releasing the soluble forms of HAI-1 but also characterization of their intracellular localization and physiological relevance are required to understand the inhibitory activity of HAI-1 against HGF activator *in vivo*.

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